

Short communication

Molecular cloning and expression of channel catfish, *Ictalurus punctatus*, complement membrane attack complex inhibitor CD59

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Abstract

The channel catfish, *Ictalurus punctatus*, complement membrane attack complex inhibitor CD59 gene was cloned and analyzed. Total RNA from tissues was isolated and cDNA libraries were constructed by the rapid amplification cDNA end (RACE) method. The gene-specific primers in conjunction with the RACE primers were used to PCR amplify 5'- and 3'-ends of the CD59 transcript. The complete channel catfish CD59 cDNA comprised 1109 bp including a 132-bp 5'-untranslated, a 360-bp open reading frame, and a 617-bp 3'-untranslated region. The open reading frame encodes a putative protein of 119-amino acid residues with calculated molecular mass (without potential glycosylation) of 13.2 kDa. However, the CD59 protein has a potential *N*-glycosylation site at the Asn35 residue. The degree of conservation of the channel catfish amino acid sequence to mammalian counterparts is 24–32%, while to those of other fish species is 44–54%. One remarkable feature is that the number and position of cysteine residues were conserved in the mature protein among species examined, suggesting that although the primary amino acid sequences are divergent, the three-dimensional structure of CD59 via disulfide linkages may be conserved through the evolutionary process. The putative protein could be further divided into three domains: a 21-amino acid signal peptide at the N-terminus, a 72-amino acid mature protein, and a 26-amino acid glycosylphosphatidylinositol (GPI) anchoring signal peptide at the carboxyl terminus. CD59 was expressed in all channel catfish tissues studied, suggesting that like mammals, channel catfish CD59 is constitutively expressed.

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Keywords: CD59; Channel catfish; *Ictalurus punctatus*; Complement membrane attack complex inhibitor

1. Introduction

The complement system plays a critical role in innate immunity. After activation by either the classical, lectin or alternative pathway, the complement system undergoes a cascade of reactions leading to opsonization and/or killing of invading pathogens (Cole and Morgan, 2003; Kim and Song, 2006; Longhi et al., 2006).

Despite its beneficial functions in protection, complement can sometimes cause host tissue damage resulting in immunological diseases (Cole and Morgan, 2003; Mollnes et al., 2002). Thus, complement activation is tightly controlled. In human and mouse, a family of cell membrane-associated proteins, which is involved in regulation of complement activation has been identified (Kim and Song, 2006; Longhi et al., 2006). CD59 is one of the members in this family.

CD59, also known as protectin (Meri et al., 1990a,b), is an 18–20 kDa glycosylphosphatidylinositol-anchored membrane glycoprotein (Davies et al., 1989; H. Okada

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et al., 1989; N. Okada et al., 1989; Sugita et al., 1988). CD59 prevents membrane lysis by binding to the α -chain of C8 in the C5b-8 complex and to the b domain of C9, and thereby inhibits the pore formation in the membrane attack complex (C5b-9) (Meri et al., 1990a; Ninoyima and Sims, 1992; Rollins and Sims, 1990; Rollins et al., 1991). In fish, Lee and Goetz (1998) identified the CD59 gene in phorbol ester-stimulated ovarian tissue from brook trout (*Salvelinus fontinalis*), but its biological function is yet to be determined.

In the course of studying the pathogenesis of *Edwardsiella ictaluri* (etiologic agent of enteric septicemia of catfish), we found that CD59 gene expression was up-regulated during *E. ictaluri* infection in channel catfish ovary cell culture (unpublished observation). Our interest in gene expression in infectious disease prompted us to characterize the CD59 gene in channel catfish. In this communication, we reported the isolation and characterization of channel catfish CD59.

2. Materials and methods

Channel catfish (NWAC 103 strain, weighed 25–30 g) were used in this study. The protocol for animal usage in experiments was approved by the Institutional Animal Care and Use Committee, Aquatic Animal Health Research Unit, Mid-South Area, Agricultural Research Service, United States Department of Agriculture.

Total RNA from spleen, head kidney, liver, intestine, skin, and gill was isolated by using Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instruction. The quality and quantity of the isolated RNA were determined by an Agilent Bioanalyzer using RNA 1200 chips (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instruction. Both 18S and 28S RNA bands were clearly identified.

After total RNA isolation, cDNA libraries were generated using the GeneRacer kit (Invitrogen, Carlsbad, CA) and following the protocol provided. Channel catfish CD59 cDNA transcript was PCR amplified using the primers listed in Table 1. The PCR products were analyzed by electrophoresis with 2% agarose gels and cloned into the pCR4-TOPO TA cloning vector (Invitrogen) that was subsequently transformed and propagated in *E. coli*.

The DNA sequencing of plasmid inserts was performed at the USDA ARS Mid-South Area Genomics Laboratory (Stoneville, MS) with Big Dye terminator chemistry version 3.1 on an ABI3730xl DNA analyzer (Applied Biosystems, Foster City, CA). More than 20 clones of each PCR product were sequenced in both strands.

Sequence chromatograms were edited for quality and vector-trimming by using the Phred (Ewing and Green, 1998; Ewing et al., 1998) and Lucy (Chou, 1998) softwares. The nucleotide and deduced amino acid sequences were aligned by using ClustalW (v.1.83) (Chenna et al., 2003), and the phylogenetic and molecular evolutionary analyses were conducted by using MEGA (v. 3.1) (Kumar et al., 2004). The hydrophobicity values of signal peptides were calculated by the method of Hopp and Woods (1981).

Real-time RT-PCR analyses of CD59 gene expression were carried out by a two-step procedure. First, cDNA libraries were generated using Superscript reverse transcriptase (Invitrogen). A TaqMan DNA polymerase PCR Master Mix kit (Applied Biosystems) was used according to the manufacturer's instruction. The amplification was carried out on an ABI PRISM 7000 SDS system with following conditions: 50 °C for 2 min, 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for one min. Relative expression data was calculated by the method

Table 1
Oligonucleotides used for PCR amplification in this study

Oligonucleotide	Direction	Sequence	T_m (°C)
GeneRacer 5' Primer (Invitrogen)	Forward	5'-CGACTGGAGCACGAGGACACTGA-3'	74
GeneRacer 3' Primer (Invitrogen)	Reverse	5'-GCTGTCAACGATACGCTACGTAACG-3'	78
CD59-40F	Forward	5'-GCCCTATTGGGCTGGGGTCTGCTAT-3'	77
CD59-287F	Forward	5'-CTGTGAGCGCAGCCAGGACTTCAGTG-3'	77
CD59-101R	Reverse	5'-CAGCCGTCATTATGGCCAC-3'	58
CD59-298R	Reverse	5'-CTGCGCTCACAGACGCACCGTTACAC-3'	77
β -Actin-F	Forward	5'-CACCATTGGCAATGAGAGGTTC-3'	68
β -Actin-R	Reverse	5'-CCTTCTGCATCCTGTCTGCAA-3'	68
CD59-Probe		5'-VIC-TAGACTACACCGGCAAATGCAATAACACCA-TAMRA-3'	
β -Actin-Probe		5'-FAM-CAACACTGTACTGTCTGGCGGTACCACCAT-TAMRA-3'	

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aaggcgtggc cagatgacgg ctgttggcga atctggtgat cacagacaca gagctctcag atcagtgaga 70

aagcagtggg taattagggtt cttgaggagc gtctgataaa acggaagacc acagggtgca gg atg M K V aaa gtt 141

F V Q V S V V F V L A L I G L G S A I K C
ttt gtg caa gtg agc gtg gta ttt gtt ctg gcc ctt att ggg ctg ggg tct gct att aag tgc 204

Y N R V D Y T G K C N N T I H C G G H N D
tac aat cgt gta gac tac acc ggc aaa tgc aat aac acc att cac tgt ggt ggc cat aat gac 267

G C L I L R E R N G K I Y R Q C I R Y S D
ggc tgc ctg ata ctg aga gag aga aat ggg aaa ata tac cgg cag tgt atc cgg tat tca gac 330

C K S A I L S T M F P H V A S F T H D C C
tgt aaa tct gct atc ctt agt acc atg ttt ccc cat gtg gcc agc ttc act cac gac tgc tgc 393

D K D L C N G A S V S A A R T S V M A M L
gac aaa gac ttg tgt aac ggt gcg tct gtg agc gca gcc agg act tca gtg atg gcc atg ctt 456

L S L A L F W W C I I *
ctt tct ctg gct ctg ttc tgg tgg tgc atc att taa tcaaacatgt tcggatactg ccctctgata 522
ctgggcatag caatactagc cctcagtgtt ttacttttaa ggtgttcaag attttaaata aataaacaga 592
caaactacag agaccgcctt tttttgtaat agcacaatgt caacagattc atagtattaa taaaatgaat 662
acttcacatt aatggtatgt aaatttaagg tatatatattt cagttgatgt taaatttgct ctgaggaagt 732
gaaattgtat aggttacatt atttttatc tcattaaaaat gtattaattt atttattcat tactttatag 802
agttaatact ggtaacatgg ttggatactg tatgtgcact aacatctgta caaaatgact ccattttata 872
tagtattcaa tactaatggc attattggct ttattctgtt agaggagtgt aactcgggct ctccttaacc 942
atggactttg gttttggaac ttcaaacaaa aggagaagtc atcttttaca ttgaacagaa gtcctttaca 1012
ttgggctttt cccccaagac tagtcttcca gtactaataa gtgtacatcc aataaaggaa gcaactttta 1082
actcaaaaaa aaaaaaaaaa aaaaaaa 1109

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Fig. 1. Complete cDNA (lower case) and deduced amino acid (upper case in the one-letter amino acid code) sequences of channel catfish CD59. Start and stop codons are indicated by bold letters. (*) Stop codon.

of $2^{\Delta\Delta C_T}$ (Livak and Schmittgen, 2001). Expression of β -actin gene was used as an internal control for normalization.

The complete channel catfish CD59 cDNA nucleotide sequence was deposited in the GenBank database and its accession no. is DQ863511.

3. Results and discussion

Previously, we performed suppression-subtractive hybridization to partially identify the channel catfish complement membrane attack complex inhibitor CD59 (unpublished data). Based on this partial CD59

Table 2
Amino acid sequence identity of CD59 among species in percentage

	Channel catfish	Pig	Cattle	Dog	African green monkey	Human	Rabbit	Norway rat	Mouse	Chicken	Zebrafish	Tetraodon nigroviridis	Rainbow trout
Channel catfish													
Pig	28.0												
Cattle	24.4	60.2											
Dog	32.5	53.7	48.0										
African green monkey	31.3	51.1	46.5	47.3									
Human	24.2	46.6	47.3	46.5	84.4								
Rabbit	27.6	50.8	41.4	44.8	45.7	47.7							
Norway rat	26.5	47.7	39.4	41.0	47.7	46.2	47.7						
Mouse	26.7	40.2	35.9	37.1	44.4	40.7	42.4	69.0					
Chicken	24.4	26.8	25.0	33.3	35.2	32.8	33.3	34.1	32.1				
Zebrafish	53.8	28.2	32.0	25.8	27.7	26.9	25.7	27.3	24.6	27.6			
Tetraodon nigroviridis	46.2	25.6	24.4	28.2	23.8	24.6	26.3	31.1	28.7	25.8	54.2		
Rainbow trout	44.5	28.0	25.8	23.4	28.1	29.7	27.9	31.1	27.9	26.8	46.2	53.8	

Identity between two species was calculated by the Blosum62 matrix of the Needle program (via <http://www.ebi.ac.uk>).

sequence, we designed gene-specific primers in conjunction with the GeneRacer primers (Table 1) to PCR amplify the 5'- and 3'-ends of the transcript. The complete channel catfish CD59 cDNA nucleotide comprised 1109 bp including a 132-bp 5'-untranslated region, a 360-bp open reading frame region, and a 617-bp 3'-untranslated region (Fig. 1). The channel catfish CD59 cDNA was shorter than those of human (1152 bp) (Davies et al., 1989), mouse (1278 bp) (Powell et al.,

1997) and rat (1540 bp) (Rushmere et al., 1994), but longer than the brook trout (*S. fontinalis*) (652 bp) CD59 gene (Lee and Goetz, 1998). The differences in cDNA sequence mainly occurred in various lengths of 3'-untranslated region.

In the 5'-untranslated region, the channel catfish CD59 cDNA contained the Kozak sequence (A/G NNATG) that is required for ribosomal recognition and protein expression (Kozak, 1987). This Kozak sequence

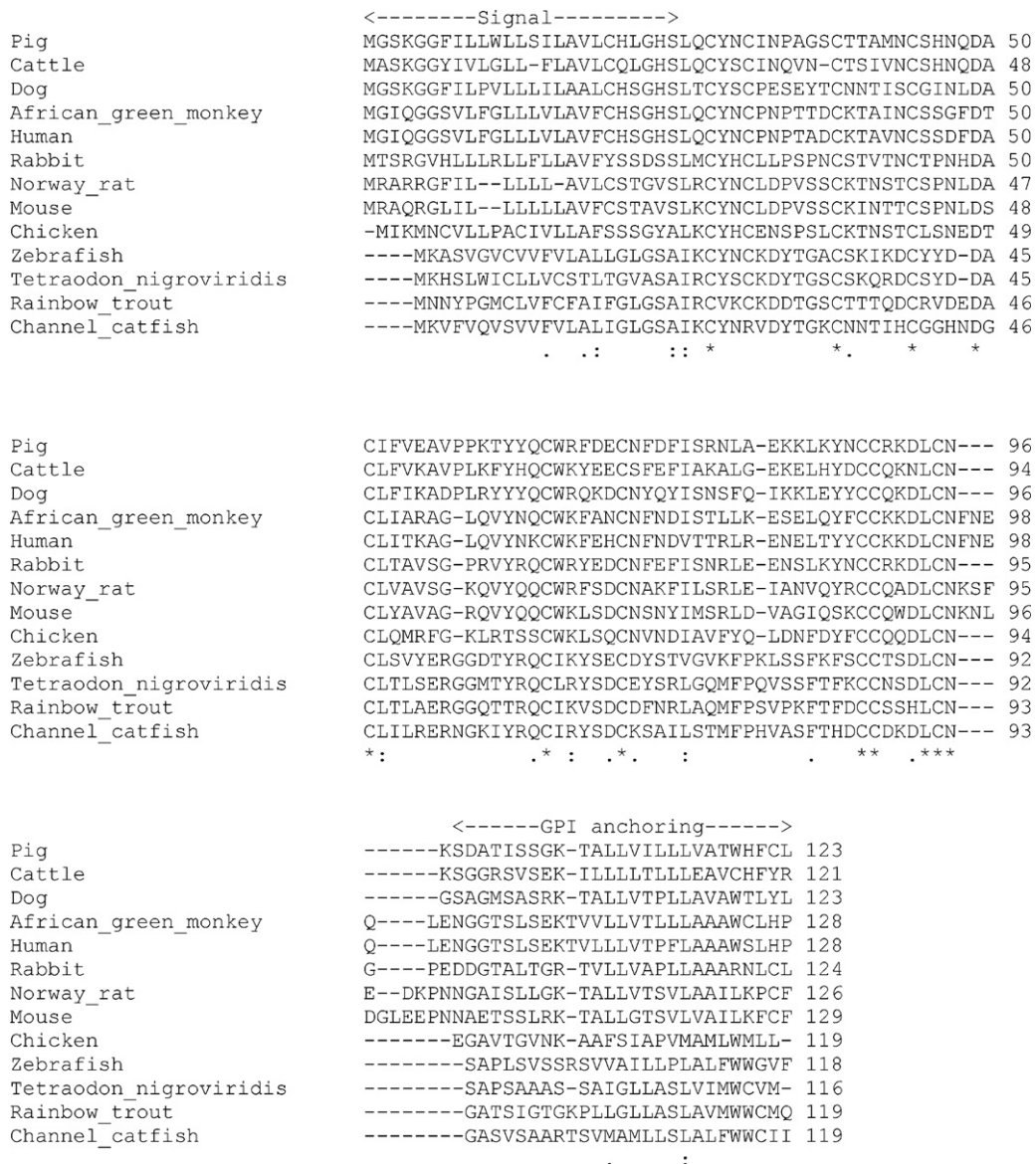


Fig. 2. Alignment of the deduced amino acid sequence of channel catfish CD59 with other CD59 amino acid sequences deposited in the GenBank database. To optimize maximal alignment, gaps were introduced in the sequences indicated as (-). Identity and similarity of amino acids are indicated by (°) and (: or .), respectively. Accession numbers of each sequence are as follows: African green monkey, Q28216; cattle, AA108201; channel catfish, DQ863511; chicken, XP_421075; dog, XP_533156; human, NP_976075; mouse, AAL04434; Norway rat, NP_037057; pig, NP_999335; rabbit, AAC23590; rainbow trout, AAT94063; Tetraodon nigroviridis, CAG01322; and zebrafish, XP_692374.

is also found in pig CD59 (Hinchliffe et al., 1998) and rat CD59 (Rushmere et al., 1994). Four consensus polyadenylation signals (aataaa) at positions 579–584, 583–588, 651–656 and 1063–1068, and a 23-nucleotide polyadenylation tail were identified in the 3'-untranslated region. The open reading frame encodes a putative protein of 119-amino acid residues with calculated molecular mass (without potential glycosylation) of 13.2 kDa, which is close to rat CD59 molecular mass of 13.8 kDa (Rushmere et al., 1994). In addition, the deduced channel catfish CD59 amino acid sequence had one potential *N*-glycosylation site at the Asn35 residue, which is similar to rat CD59 at the Asn38 residue and corresponds to the Asn79 residue of human CD59 (Fig. 1).

To compare the deduced channel catfish CD59 amino acid sequence with the known CD59 sequences

deposited in the GenBank database, the sequences were aligned by using ClustalW (Chenna et al., 2003). The degree of conservation of the channel catfish CD59 sequence to mammalian counterparts is 24–32%, while channel catfish CD59 to that of other fish species is 44–54% (Table 2). One striking feature is that the number and position of cysteine (Cys) residues were conserved among all species examined (indicated as (*) in Fig. 2), suggesting that although the primary amino acid sequences are divergent, the three-dimensional structure of CD59 via disulfide linkages (nine Cys residues conserved) may be conserved through the evolutionary process (Fletcher et al., 1994; Rushmere et al., 1994).

When compared with human CD59 (Fletcher et al., 1994), the channel catfish CD59 amino acid sequence showed that its sequence could be further divided into three domains: a 21-amino acid signal peptide at the

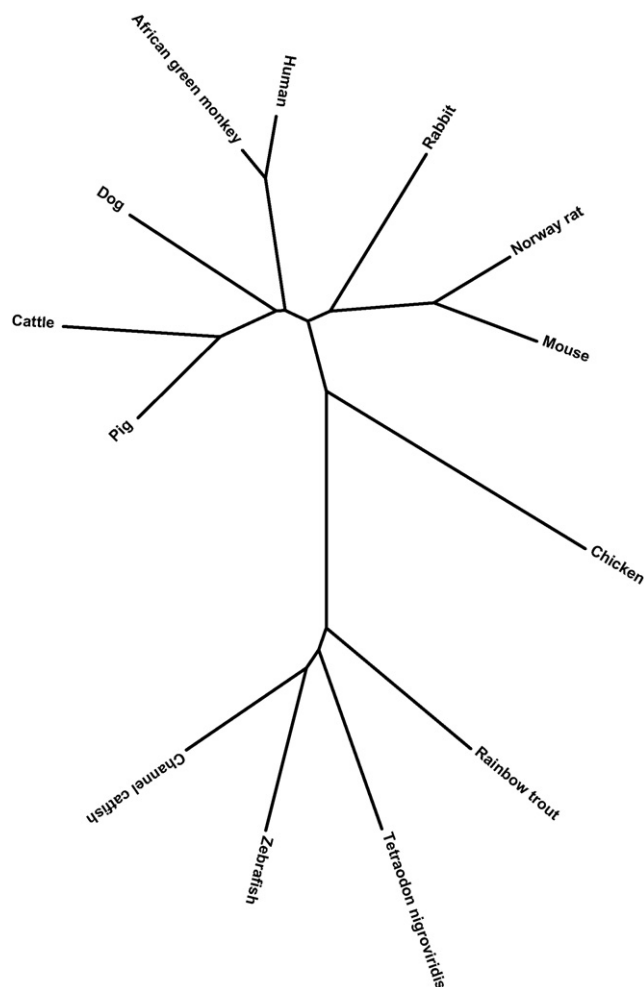


Fig. 3. Molecular phylogenetic relationships of CD59 amino acid sequences. Sequences from Fig. 2 were used to generate the phylogenetic tree by bootstrap analysis (1000 replications) of the Neighbor-Joining method in the MEGA3 (v.3.1) program (Kumar et al., 2004).

N-terminus with hydrophobic residues, a 72-amino acid mature protein, and a 26-amino acid glycosylphosphatidylinositol (GPI) anchoring signal peptide at the carboxyl terminus with hydrophobic residues (Fig. 2; Table 3). In the mature protein, fish started with the conserved Ile22 residue, while mammalian CD59 started with the conserved Leu residue (Fig. 2). This is because a single nucleotide was different in fish (att) compared to mammals (ctt). In addition, although amino acid sequences of signal peptides are different, they have the similar hydrophobic property and thus may have the same function as a signal (Table 3) (Gerber et al., 1992; Yeh et al., 1996).

To determine evolutionary relatedness of CD59 among animals, a phylogenetic evolutionary tree was

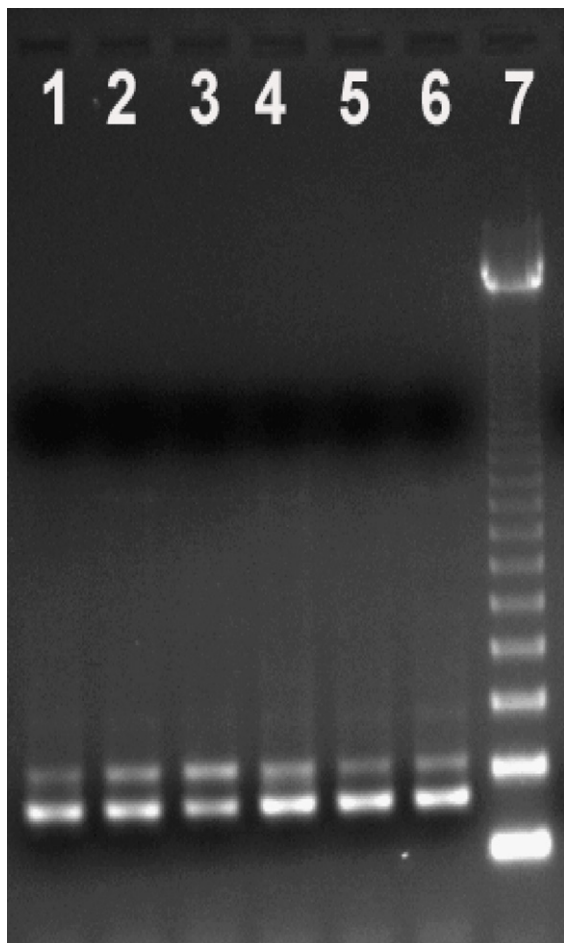


Fig. 4. Tissue distribution of channel catfish CD59 gene. Total RNA from channel catfish tissues were prepared for RT-PCR. The amplified products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide. Lanes 1, spleen; 2, head kidney; 3, liver; 4, intestine; 5, skin; 6, gill; and 7, 123 bp molecular standards (Invitrogen). CD59 and β -actin are located at upper bands (259 bp) and lower bands (210 bp), respectively.

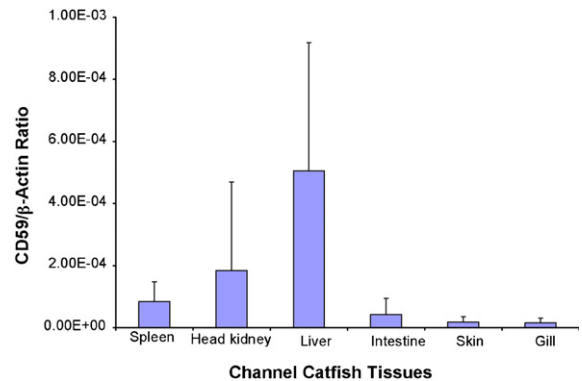


Fig. 5. Tissue distribution of channel catfish CD59 gene determined by quantitative RT-PCR with a TaqMan DNA polymerase PCR Master Mix kit ($n = 3$ healthy fish).

constructed by the Neighbor-Joining method based on the ClustalW alignment (Kumar et al., 2004). As seen in Fig. 3, fish CD59 formed a distinguishable clade from that of mammalian counterparts. However, we do not know whether fish, with more than 20,000 species (Helfman et al., 1997), form a well-supported clade. In our recent study on channel catfish hemoglobin- β , we demonstrate that a high degree of diversity of hemoglobin- β among fish (22 out of 148-amino acid residues are conserved throughout the sequences) (Yeh et al., 2006). The tree also shows the phylogenetic transition from water-living fish to bird to land inhabitants.

The expression profile of CD59 was examined in channel catfish spleen, head kidney, liver, intestine, skin and gill with multiplex RT-PCR amplification. The primer set of CD59-40F and CD59-298R was used to amplify the CD59 transcript, and the primer set of β -Actin-F and β -Actin-R was for β -actin amplification. As seen in Fig. 4, RT-PCR products of both β -actin (210 bp, lower bands) and CD59 (259 bp, upper bands) were detected from different channel catfish tissues. For further quantitative analysis of the expression of CD59 transcript, a two-step real-time RT-PCR assay was used. Comparison of cycle threshold of β -actin and CD59 transcripts showed that CD59 expression was in lower abundance than β -actin gene in tissues examined. As seen in Fig. 5, the different levels of expression of CD59 transcript among channel catfish tissues were observed. These results suggest that fish CD59 is constitutively expressed. In addition, results from our study as well as other's group (Lee and Goetz, 1998) demonstrate a high level of CD59 expression in liver. One explanation is that hepatocytes are the primary site for complement synthesis and, thus, a tight regulation of the system is

Table 3

Amino acid sequences and hydrophobicity values of signal peptides and GPI-anchoring signal of CD59 protein

Species	Amino acid sequence ^a	Hydrophobicity value ^b	Peptide
Channel catfish	NH ₂ -MKVVFQVSVVFLALIGLGS-COOH	−19.7	Signal peptide
Zebrafish	NH ₂ -MKASVGVCVFLALLGLGS-COOH	−17.4	Signal peptide
Chicken	NH ₂ -MIKMNCVLLPACIVLLAFSSSGYA-COOH	−20.6	Signal peptide
Pig	NH ₂ -MGSKGGFILLWLLSILAVLCHLGH-COOH	−23.5	Signal peptide
Mouse	NH ₂ -MRAQRGLILLLLLAFCSTAVS-COOH	−16.5	Signal peptide
Norway rat	NH ₂ -MRARRGFILLLLLAFCSTGVS-COOH	−12.2	Signal peptide
Human	NH ₂ -MGIQGGSVLFGLLLVAVFCHSGHS-COOH	−23.0	Signal peptide
Channel catfish	NH ₂ -ASVSAARTSVAMLLSLALFWWCII-COOH	−25.4	GPI-anchoring
Zebrafish	NH ₂ -APLSVSSRSVAILLPLALFWWGVF-COOH	−25.9	GPI-anchoring
Chicken	NH ₂ -GAVTGVNKAAFSIAPVMAMLMWLL-COOH	−20.9	GPI-anchoring
Pig	NH ₂ -SDATISSGKTALLVILLVATWHFCL-COOH	−20.6	GPI-anchoring
Mouse	NH ₂ -NNAETSSLRKTALLGTSVLVAILKFCF-COOH	−9.2	GPI-anchoring
Norway rat	NH ₂ -NNGAISLLGKTALLVTSVLAAILKPCF-COOH	−16.7	GPI-anchoring
Human	NH ₂ -NGGTSLSSEKTVLLLVTPFLAAWSLHP-COOH	−15.8	GPI-anchoring

^a References to channel catfish (GenBank accession no. DQ863511), zebrafish (XP_692374), chicken (XP_421075), pig (NP_999335), mouse (AAL04434), Norway rat (NP_037057) and human (NP_976075).

^b Calculated by the method of Hopp and Woods (1981).

necessary for prevention of immunological diseases caused by the complements.

In conclusion, we isolated and characterized the channel catfish CD59 gene. This information will further help to elucidate the complex channel catfish innate immune system. Further experiments to express channel catfish CD59 in *E. coli* and raise polyclonal antibodies against the CD59 peptide are underway.

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